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Nitrite modulates contractility of teleost (*Anguilla anguilla* and *Chionodraco hamatus*, i.e. the Antarctic hemoglobinless icefish) and frog (*Rana esculenta*) hearts

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ABSTRACT

Being the largest form of intravascular and tissue storage of nitric oxide (NO) and a signalling molecule itself, the nitrite anion (NO_2^-) has emerged as a key player in many biological processes. Since the heart is under an important NO-mediated autocrine–paracrine control, in mammals the cardiac effects of nitrite are under intensive investigation. In contrast, nothing is known in non-mammalian vertebrates. We evaluated nitrite influence on cardiac performance in the perfused beating heart of three different cold-blooded vertebrates, i.e. two teleost fishes, the temperate red-blooded *Anguilla anguilla*, the Antarctic stenotherm, hemoglobinless *Chionodraco hamatus* (icefish), and the frog *Rana esculenta*. We showed that, under basal conditions, in all animals nitrite influences cardiac mechanical performance, inducing negative inotropism in eel and frog, while being a positive inotrope in *C. hamatus*. In all species, these responses parallel the inotropic effects of authentic NO. We also demonstrated that the nitrite-dependent inotropic effects are i) dependent from NO synthase (NOS) activity in fish; ii) sensitive to NO scavenging in frog; iii) cGMP/PKG-dependent in both eel and frog. Results suggest that nitrite is an integral physiological source of NO and acts as a signalling molecule in lower vertebrate hearts, exerting relevant inotropic actions through different species-specific mechanisms.

1. Introduction

Over recent years, the biology of inorganic nitrogen oxides has been the subject of many investigations. Although knowledge on their biological effects of nitrite has a long and rich history, the emerging role of nitrite as a nitric oxide (NO) reservoir in mammals, including man, opened the door to novel implications both at physiological and pathological levels [1,2].

Nitrite anion (NO_2^-) is relatively abundant in blood and tissues and its plasma levels are conserved across various mammalian species, including man, in the range of 150–600 nmol l^{-1} and may increase slightly in response to inflammation or infection [3,4]. It was estimated that as much as 70% of plasma nitrite originates from nitric oxide synthases (NOSs), mainly in the endothelium by endothelial NOS (eNOS). In fact, nitrite has been reported as an index of NOSs activity [1]. In addition to endogenous generation, exogenous sources, principally environmental pollutants and intake of vegetables, contribute to the build-up of this NO reserve [5].

A growing body of evidence suggests that nitrite anion, rather than being an inert waste product of NO metabolism, may be a key player in biological processes, representing, on one hand, the largest form of intravascular and tissue storage pool of NO and, on the other hand, a signalling molecule itself [6].

Mechanisms for the *in vivo* conversion of nitrite to NO may involve either enzymatic reduction or non-enzymatic reduction [see2 for references]. Some proteins show nitrite reductase capacity, i.e. glutathione-S-transferases, xanthine oxidoreductase, cytochrome P-450 enzymes, deoxy-hemoglobin and deoxy-myoglobin, as well as eNOS [7,8]. Each mechanism would occur preferentially during pathological hypoxia and acidosis[9] present in disease states, such as ischemia [10]. In fact, during ischemia this alternative NO production may exert a protective influence because L-arginine-NOS-derived NO generation depends on oxygen, which is rapidly depleted under ischemic conditions. Accordingly, recent evidence is consistent with a powerful protective influence of nitrite in animal models of ischemia–reperfusion injury [10,11]. It was suggested that nitrite acts via formation of NO, but there is also a possibility that nitrite can modulate physiological processes via other pathways, for example through direct S-nitrosylation of thiol-containing proteins.

Recently, some authors have reported that nitrite has a distinct and important signalling role under normal physiological conditions, being capable of modulating many important signalling pathways, including soluble guanylyl cyclase (sGC) stimulation, cytochrome P-450 activity and the expression of two archetypical proteins, heat shock protein 70 (Hsp 70) and heme oxygenase-1 (HO-1) [12]. Nitrite

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is a quantitative relevant component of the environment of cold-blooded vertebrates, particularly fish and amphibians, and therefore we may expect that in these animal groups the anion-elicited effects may be, at least, as important as in mammals. Furthermore, many fish and amphibians are chronically exposed to the challenge of environmental hypoxia and acidosis. For the first time in fish, Jensen [13] tested the hypothesis that NO_2^- accumulation across the gills of zebrafish causes a relevant production of NO from nitrite. He used deconvolution spectral methodology and blood level of nitrosylhemoglobin (HbNO), as a biomarker of intra-organismal NO generation, and showed very high HbNO levels in fish exposed for variable time periods to different doses of nitrite (dissolved as $NaNO_2$ in the environmental water). This finding is consistent with deoxyHb-mediated reduction of nitrite as a major NO-producing mechanism which appears physiologically important in the *in vivo* arterial-venous circulation when Hb circulates between full and intermediate oxygen saturation. The aim of this research was to explore the biological activity of nitrite as a putative signalling molecule under basal conditions in isolated and perfused working hearts (teleost fishes and frog), selected as appropriate case study models of lower vertebrate cardiac physiologies.

We used the temperate eurytherm European eel (*Anguilla anguilla*) and the cold-adapted Antarctic *Chionodraco hamatus* (icefish) and the frog *Rana esculenta*. Both eel and frog experience considerable fluctuations of environmental oxygen, the former being a champion of prolonged hypoxia and acidosis tolerance. In contrast, the icefish represents a unique case of extreme stenothermia; in fact, it is an endemic inhabitant of the stably icy and richly oxygenated Antarctic waters. Conceivably, these environmental conditions have allowed the animal to survive despite its paradoxical loss of hemoglobin and red blood cells, normally indispensable for adult vertebrate life [see 14,15 for references]. Since Hb is a key protein in NO homeostasis, functioning both as NO scavenger and generator from nitrite, the hemoglobinless icefish provides exclusive opportunities to investigate NO/nitrite signalling in a naturally occurring genetic knockout for Hb. This comparative analysis may be of relevance to understand the biological activity of nitrite in modulating the "normal" function of the vertebrate heart, verifying, at the same time, to which extent aspects of cardiovascular regulation and even classification of NO and nitrite-dependent mechanisms can be applied to representatives of more than one group (mammals) of vertebrates.

2. Materials and methods

2.1. Chemicals

Biotin-HPDP was purchased from Pierce; ECL was purchased from Amersham; the other chemicals/drugs were purchased from Sigma unless otherwise indicated and prepared immediately before each experiment. ODQ was prepared in ethanol, while the other solutions were prepared in double-distilled water. KT5823 was used in a darkened perfusion apparatus to prevent degradation.

2.2. Animals

European eels (*A. anguilla L.*) and frogs (*R. esculenta*) of both sexes, weighing 90–120 g and 15–25 g respectively, were provided by a local hatchery and kept at room temperature (18–20 °C) without feeding for 5–7 days.

Male and female icefish (*C. hamatus*), weighing 320–370 g, were caught by nets in the Terranova Bay, Ross Sea, Antarctica during January 2005. The animals were maintained unfed for at least 5 days in aerated, running seawater at temperatures between 0 and 2 °C. The experiments were done in the Italian Antarctic Base laboratories, Terranova Bay (74°42′S,164°06′E), during the XX Italian Antarctic Expedition (December 2004–February 2005).

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Isolated and perfused working heart preparations

Both fish (eel and icefish) were anaesthetized in benzocaine (0.2 g l^{-1}) for 15 min and were opened ventrally. The ventral aorta was cannulated and the heart removed without the pericardium and placed in an ice-chilled dish filled with saline for the atrium cannulation procedure. A polyethylene cannula was secured in the atrium at the junction with the *sinus venosus*. Isolation time was 5 min.

Frogs were pited and ventrally opened; the pericardium was removed and the heart cannulated *in situ* with polyethylene cannula inserted into the *sinus venosus* for the inflow and into the left aortic trunk for outflow collection. Right aortic trunk, right and left *praecava* veins and pulmonary vein were tied off by ligature. Isolation time was 15–20 min.

The cannulated heart was transferred to a perfusion chamber filled with saline and connected with a perfusion apparatus as previously described [eel, 16; icefish, 17; frog, 18]. Perfusion was immediately started; the heart received saline from an input reservoir pumped against an afterload pressure given by the height of an output reservoir.

The hearts were perfused with Ringer's solution at constant input pressures and were able to produce physiological values of work and power [eel, 16; icefish, 17; frog, 18]. The experiments on eel and frog were performed at room temperature (18–20 °C), the experiments on icefish were performed in a cold thermostatted cabinet (LKB 2021 Maxicoldlab, Malbo, Sweden), which allowed the heart and perfusion system to be maintained at near zero temperatures. The control conditions were: *A. anguilla*, mean output pressure 3.00 kPa with cardiac output set to 10 ml min^{-1} kg^{-1} body mass; *C. hamatus*, mean output pressure 1.4 kPa with cardiac output set to 50 ml min^{-1} kg^{-1} body mass; *R. esculenta*, mean output pressure 3.92 kPa with cardiac output set to 110 ml min^{-1} kg^{-1} body mass. Cardiac output was set by appropriately adjusting filling pressure.

Heart rate (HR) was calculated from pressure recording curves. Cardiac output (CO) was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. Stroke volume (Vs; ml kg^{-1}; CO/HR) was used as a measure of ventricular performance; changes in Vs were considered to be inotropic effects. CO and Vs were normalized per kilogram of wet body mass. Ventricular stroke work [Ws; mJ g^{-1}; (afterload–preload)×Vs/ventricle mass] served as an index of systolic functionality. Ws was corrected for the ventricle weights (eel, 0.065 ± 0.003; icefish, 1.12 ± 0.23; frog, 0.048 ± 0.002) measured at the end of each experiment after the removal of excess water.

2.4. Experimental protocol

In all experiments, hearts were allowed to equilibrate to conditions which simulate an *in vivo* resting state for up to 15–20 min. Hearts that did not stabilize within 20 min of perfusion were discarded. Cardiac variables were measured simultaneously during experiments. All experiments were carried out on the basis of endurance profile for each species (eel, 120 min, [16]; icefish, 120 min, [17]; frog, 80 min, [18]). To avoid the influence of chronotropism on inotropism, all the hearts were electrically paced at spontaneous rhythm. However, control experiments on unpaced heart (data not shown) revealed that nitrite did not modify HR.

The response of the hearts to pharmacological agents (L-arginine: eel 10^{-7} mol l^{-1}, icefish 10^{-6} mol l^{-1} [17], frog 10^{-7} mol l^{-1}; L-NMMA: 10^{-5} mol l^{-1}; SIN-1: eel 10^{-7} mol l^{-1}, icefish 10^{-5} mol l^{-1} [17], frog 10^{-7} mol l^{-1}; SOD: 10 IU/ml; L-NIO: 10^{-5} mol l^{-1}; PTIO:

Table 1
Baseline cardiac parameters in working hearts.

Species	Heart rate (beats/min)	Filling pressure (kPa)	Output pressure (kPa)	Cardiac output (ml min^{-1} kg^{-1})	Stroke volume (ml min^{-1} kg^{-1})	Stroke work (mJ g^{-1})
Eel	51.4 ± 12.2	0.07 ± 0.008	2.97 ± 0.034	10.9 ± 1.56	0.21 ± 0.09	0.105 ± 0.03
Icefish	31.2 ± 0.79	0.117 ± 0.011	1.385 ± 0.085	50.5 ± 3.62	1.861 ± 0.113	2.241 ± 0.258
Frog	55.4 ± 2.8	0.5 ± 0.05	3.76 ± 0.1	107.4 ± 3.62	1.96 ± 0.39	4.15 ± 0.32

10^{-7} mol l^{-1}; ODQ: 10^{-5} mol l^{-1}; KT5823: 10^{-7} mol l^{-1}) was obtained by perfusing each cardiac preparations with the buffer containing only one chemical at the desired concentration in the presence or absence of increasing concentrations of sodium nitrite.

2.5. Statistics

Data were expressed as the mean ± SEM. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the paired Student's *t*-test ($P<0.05$). Comparison between groups was made using a one-way analysis of variance (ANOVA) followed by Duncan's test. Differences were considered to be statistically significant for $P<0.05$.

2.6. Homogenization protocol

Hearts were homogenized on ice in 20 mmol l^{-1} Tris pH 7.5, 150 mmol l^{-1} NaCl, 1% Igepal CA 630, 0.5% Sodium Deoxycholate, 1 mmol l^{-1} EDTA, 0.1% SDS, 200 mmol l^{-1} Sodium Orthovanadate, and Protease Inhibitor Cocktail, using a polytron tissue grinder. The homogenate was centrifuged at 4 °C for 40 min at 13,000 *g*. The supernatant containing cytosolic proteins was collected and proteins were quantified with Bradford reagent; the pellet containing membrane proteins was resuspended in homogenization buffer and proteins were quantified with Bradford reagent.

2.7. Biotin switch assay and Western blot

The biotin switch assay was performed essentially as previously described [19]. Extracts were adjusted to 0.5 mg/ml of protein and equal amounts were blocked with 4 volumes of blocking buffer (225 mmol l^{-1} Hepes, pH 7.7, 0.9 mmol l^{-1} EDTA, 0.09 mmol l^{-1} neocuproine, 2.5% SDS, and 20 mmol l^{-1} MMTS) at 50 °C for 20 min with agitation. After blocking, extracts were precipitated with 2 volumes of cold (−20 °C) acetone, chilled at −20 °C for 10 min, centrifuged at 2000 *g*, 4 °C for 5 min, washed with acetone, dried out at room temperature and resuspended in 0.1 ml HENS buffer (250 mmol l^{-1} Hepes, pH 7.7, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} neocuproine, and 1% SDS) x mg of protein. Until this step, all operations were carried out in the dark. A 1/3 vol of biotin-HPDP 4 mmol l^{-1} in DMF and ascorbate 1 mmol l^{-1} were added and incubated for 1 h at room temperature. Proteins were acetone-precipitated again and resuspended in the same volume of HENS buffer.

To detect biotinylated proteins by Western blot, samples from the biotin switch assay were separated on 15% SDS-PAGE gels, transferred to PVDF membranes, blocked with non fat dried milk, and incubated with streptavidin-peroxidase diluted 1/5000 for 1 h.

Blots were developed by enhanced chemioluminescence (ECL) and were placed in a film cassette with photograph film. Films were exposed for 30 s, developed and fixed.

3. Results

3.1. Isolated and perfused working heart preparations

Under basal conditions, the hemodynamic characteristics of the isolated and perfused working heart preparations from eel, icefish, and frog mimic the physiological values of the *in vivo* animal. Baseline hemodynamic parameters of all species (heart rate, filling pressure, output pressure, cardiac output, stroke volume, and stroke work) were measured after 15–20 min of equilibration (Table 1). The icefish cardiac output shown in Table 1 is an *in vitro* value obtained by us using the preparation of isolated and perfused working heart. This value is very close to the cardiac output calculated *in vivo* (61 ml min^{-1} kg^{-1} [20]).

3.2. Nitrite modulation of inotropism

To assess the effects of nitrite on cardiac function, the isolated perfused cardiac preparations from eel, icefish, and frog were exposed to nitrite and parameters of cardiac function were measured. In control experiments repeated exposures of each heart to the same concentration of nitrite did not result in pharmacological tolerance (data not shown), therefore cumulative concentration-response curves were generated.

In both eel and frog, nitrite induced a concentration-dependent negative inotropic effect, shown by a decrease in stroke volume and

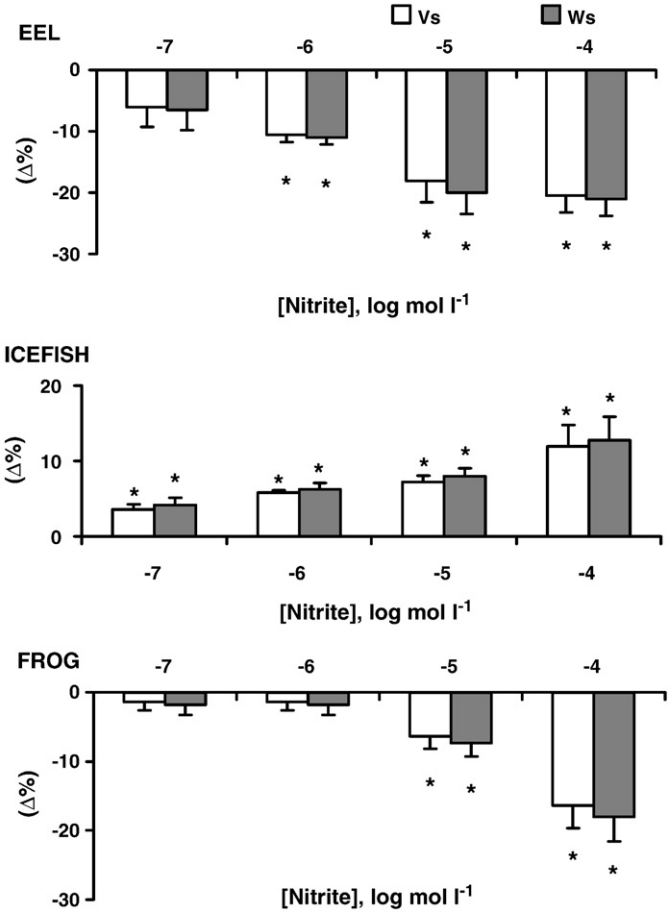

Fig. 1. Cumulative dose–response curves of nitrite on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel, icefish and frog ($n=5$). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$.

stroke work, significant from 10^{-6} mol l^{-1} in the eel and from 10^{-5} mol l^{-1} in the frog. In contrast, in the hemoglobineless icefish, increasing concentrations of nitrite positively modulated inotropism, as indicated by an increase in stroke volume and stroke work, significant from 10^{-7} mol l^{-1} (Fig. 1).

3.3. Inotropic effects induced by authentic nitric oxide

To determine the effects of endogenously generated NO on cardiac function, we treated the isolated heart preparations with the physiological substrate for NO synthase (NOS), L-arginine and a pure NOS-independent NO donor, SIN-1. We tested several doses of L-arginine and SIN-1 and we have chosen the lowest concentration which is able to induce significant effects.

As shown in Fig. 2A, similar to the effects observed with nitrite, stroke volume and stroke work were significantly decreased by L-arginine in eel and frog, while they were increased in the icefish. In all species, the L-arginine-dependent effects were inhibited by the NOS inhibitor L-NMMA (10^{-5} mol l^{-1}), confirming that the inotropic effects of L-arginine were indeed NOS dependent (Fig. 2B).

Administration of SIN-1 in the presence of superoxide dismutase, induced a negative inotropism in eel and frog and, in contrast again, a positive inotropism in icefish (Fig. 2C).

3.4. NOS-NO involvement in the nitrite-dependent inotropy

Since NO and nitrite similarly affect the heart, to clarify whether the inotropic effect of nitrite involves NOS-produced NO, we exposed the isolated hearts of all species to nitrite (10^{-5} mol l^{-1}) alone (Fig. 3A) and in the presence of the NOS inhibitors L-NMMA (10^{-5} mol l^{-1};Fig. 3B) or L-NIO (10^{-5} mol l^{-1}; Fig. 3C). These treatments abolished nitrite-dependent negative inotropism in eel and positive inotropism in icefish. In contrast, in the frog heart NOS inhibition did not modify the nitrite-induced negative inotropism (Figs. 3B, C). To test whether nitrite reduction to NO is involved in the nitrite-dependent inotropy, we exposed the isolated hearts of all species to nitrite (10^{-5} mol l^{-1}) alone (Fig. 3A) and in the presence of a NO scavenger PTIO (10^{-7} mol l^{-1};Fig. 3D). PTIO did not modify the nitrite-induced inotropism in both eel and icefish, while it abolished the negative inotropic effects exerted by nitrite in frog (Fig. 3D).

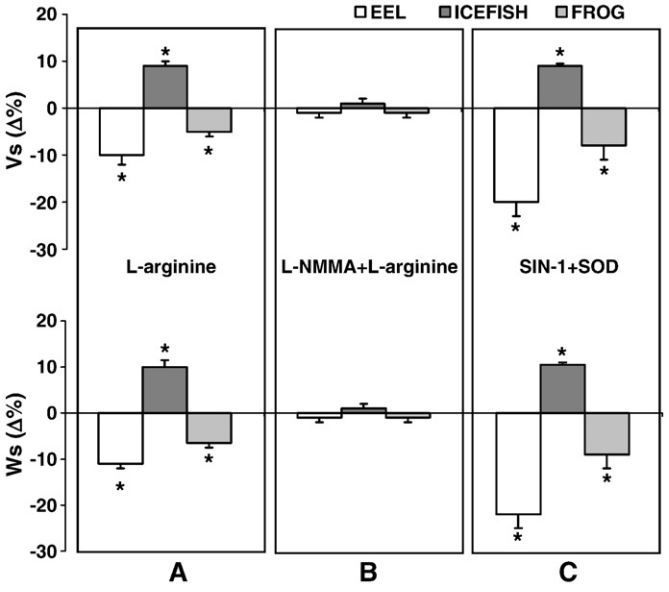

Fig. 2. Effect of L-arginine on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel (10^{-7} mol l^{-1}), icefish (10^{-6} mol l^{-1}) and frog (10^{-7} mol l^{-1}) before (A) and after (B) treatment with L-NMMA (10^{-5} mol l^{-1}) ($n=4$–7). (C) Effect of SIN-1 (in presence of SOD 10 IU/ml) on stroke volume (Vs) and stroke work (Ws) in eel (10^{-7} mol l^{-1}), icefish (10^{-5} mol l^{-1}) and frog (10^{-7} mol l^{-1}) ($n=4$–5). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$.

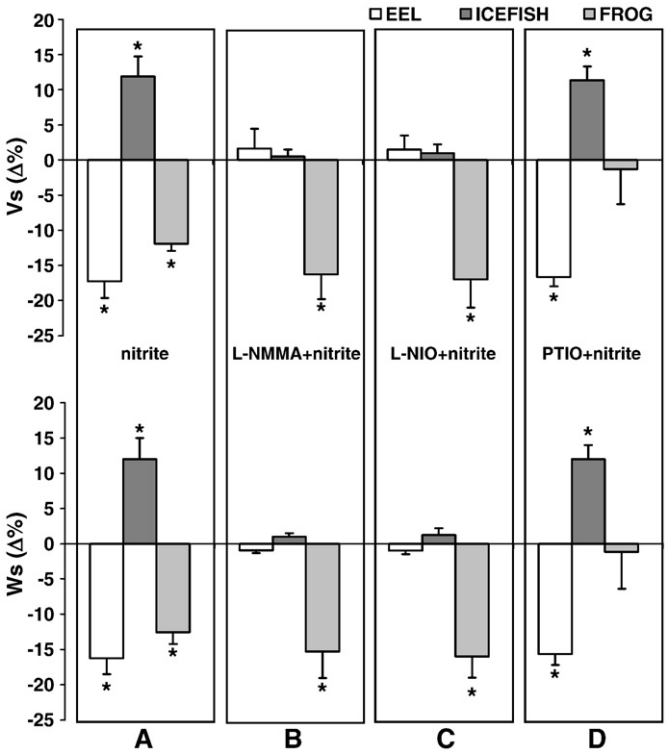

Fig. 3. Effect of nitrite (10^{-5} mol l^{-1}) on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel, icefish and frog before (A) and after treatment with L-NMMA (10^{-5} mol l^{-1}) (B) or L-NIO (10^{-5} mol l^{-1}) (C) or PTIO (10^{-7} mol l^{-1}) (D) ($n=4$–6). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$.

3.5. cGMP-PKG involvement in the nitrite-dependent inotropism

In the mammalian heart, activation of the NO-sGC pathway can mediate negative inotropy. To verify the involvement of cGMP-PKG cascade, cardiac preparations of eel and frog were perfused, in presence of nitrite, either with ODQ (10^{-5} mol l^{-1}), an oxidant and a soluble guanylate cyclase inhibitor, or with a protein kinase G blocker KT5823 (10^{-7} mol l^{-1}). (Obvious logistic difficulties in Antarctica prevented in icefish parallel experiments described in this and in the following section). As shown in Fig. 4B, in both species ODQ and KT5823 treatments abolished the negative inotropic effect of nitrite, supporting the involvement of the NO-sGC-PKG pathway in the nitrite actions (Fig. 4C).

3.6. Analysis of S-nitrosylated proteins

In the eel cardiac tissues, the analysis of S-nitrosylated proteins revealed no differences in the cytosolic fractions, while in the membrane fractions a major expression of both low and high molecular weight S-nitrosylated proteins were present (Fig. 5).

In the frog cardiac tissues, the analysis of S-nitrosylated proteins revealed differences in both the cytosolic and membrane fractions, in particularly at low molecular weight (Fig. 6).

4. Discussion

Using *in vitro* isolated and perfused working heart preparations of teleost fish, i.e. the eel *A. anguilla* and the hemoglobinless icefish *C.*

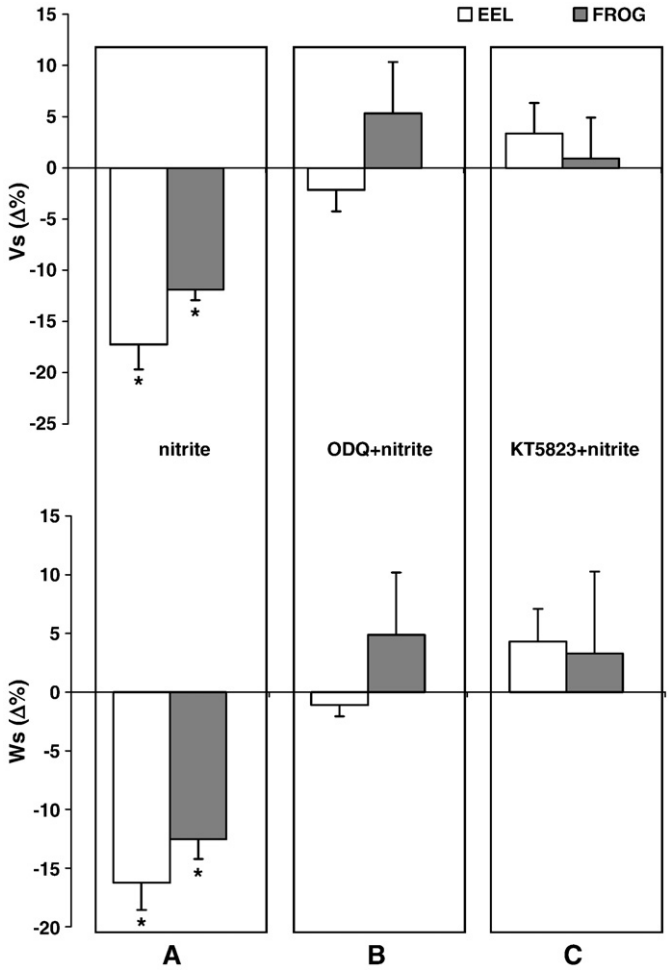

Fig. 4. Effect of nitrite (10^{-5} mol l^{-1}) on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel and frog before (A) and after treatment with ODQ (10^{-5} mol l^{-1}) (B) or KT5823 (10^{-7} mol l^{-1}) (C) ($n=3$–5). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$.

hamatus, as well as the frog *R. esculenta*, characterized by diverse life histories and ecophysiological traits, we provided heart paradigms well suited for highlighting the signalling properties of nitrite in a comparative vertebrate context. In particular, the distinct morpho-functional traits (see Table 2 for references) of these heart designs (fully trabeculated myoarchitecture supplied by avascular intertrabecular system of *lacunae*) allow assessment of nitrite-elicited contractile myocardial effects free from concomitant vascular (coronary) responses. Like in the highly vascularized mammalian heart [i.e. rat, 21], we found that nitrite exerts a remarkable contractile influence on the heart of the three species, regardless of their difference in vascular organization, i.e. avascular (frog, icefish) or poorly (eel) vascularized types of ventricles. We showed that nitrite negatively affects cardiac mechanical performance by decreasing stroke volume in eel and frog, while increasing it in the icefish. Of note, these nitrite-induced opposite myocardial effects parallel those induced by the direct cardiac exposure to NO [eel, 16; icefish, 17; frog, 18], providing a striking example of the role of nitrite reduction to NO in the heart. It is conceivable that the positive inotropic response observed in the icefish involves the hemoglobinless condition. The already documented [17] species-specific differences in the cardiac response to NO between the icefish and the eel, cannot be attributed to the experimental hierarchic level of investigation or to macroscopic differences in cardiac structure. The possibility exists that the very expanded mitochondrial compartment of the icefish myocytes [17], plays a role. In zebrafish, exposure to water nitrite (0.6–2 mmol l^{-1}) was shown to induce an increase of Hb-NO levels which indicates an augmented NO production [13]. This perturbation in NO homeostasis was suggested to be responsible for important cardiovascular effects, including vasodilation and decreased blood pressure, quickly counteracted by increased cardiac pumping [22].

Interestingly, although the icefish lacks Hb, its cardiac tissue contains myoglobin which may contribute to NO generation by functioning as nitrite reductase [23]. It is possible that the nitrite-induced positive inotropism observed in the icefish is due either to a distinct spatio-temporal sub-compartmentalization of NOS isoforms, or to intracellular effectors related to extreme stenothermia, or to the absence of Hb [17,24].

Interestingly, in contrast to the cardiac responses in eel and frog, the icefish heart shows a higher sensitivity to nitrite, the first significant increase in stroke volume being obtained at 0.1 μmol l^{-1}. In aquatic organisms, nitrite sensitivity shows large species variations [25]. As shown in many types of myocardial preparations, the shift from nanomolar to micromolar concentrations of intramyocardial NO and cGMP has been correlated to either positive or negative inotropic responses, respectively [see 26 for references]. The reason for the increased nitrite sensitivity of the icefish heart is unclear although several factors, to be mechanistically investigated in the future, may play a role, including the extreme cold adaptation and the stable oxygen-rich Antarctic waters, as well as the reactive oxygen species generating/scavenging abilities associated to the Hb-less condition.

Noteworthy, in the frog heart, the nitrite-dependent negative inotropism is inhibited by NO scavengers but not by NOS inhibitors, a result consistent with the reduction of nitrite to NO though a NOS-independent mechanism, as reported in the rat [21]. Contrarily, in both eel and icefish, NOS inhibition abolishes the nitrite-induced inotropic effects, which, however, are not affected by the NO scavenger PTIO, thus suggesting that nitrite action is not via its reduction to NO. Indeed, a mammalian-oriented study has suggested that nitrite may also mediate effects independent of NO generation [12]. The mechanistic explanation of the different pattern between fish and frog remains to be clarified. Since in fish a functional NOS appears essential for the inotropic effects exerted by nitrite, the possibility exists that in these piscine species the NOS isoforms, particularly eNOS, may act as nitrite-reductase, as suggested in some mammals [7].

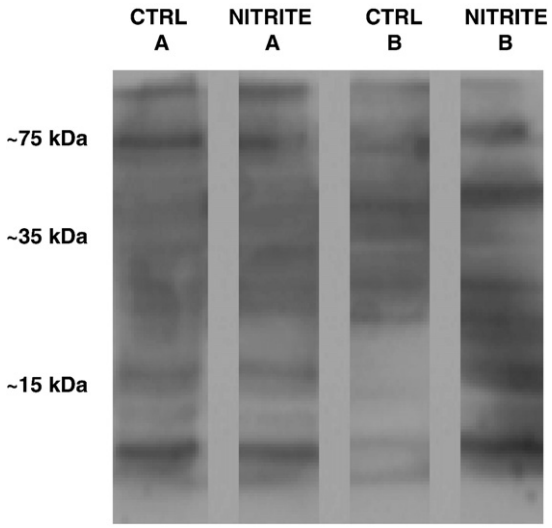

Fig. 5. Western blot analysis of S-nitrosylated proteins in eel heart homogenates. Cytosolic (A) and membrane protein fractions (B). CTRL: control; NITRITE: nitrite treated-hearts.

It was previously demonstrated that the hearts of eel, icefish and frog have a functioning NO axis which modulates mechanical performance via a NO-dependent increase in cGMP [16–18]. Also in the mammalian heart, several studies have demonstrated a NO-cGMP-dependent modulation of inotropism [26–29]. In all cases, the NOS system has been regarded as the exclusive physiological source of NO. However, here we show that in the heart of eel, icefish and frog, physiological levels of nitrite may also be integral sources of NO, thus strongly supporting the generalized picture emerged in mammalian-oriented studies. Noteworthy, as described in mammals, nitrite conversion to NO may occur along oxygen and pH gradients [see 30 for references], a condition which can be of importance in fish and amphibians, often exposed to chronic environmental hypoxia [31,32].

In eel and frog, the inhibition of the nitrite-dependent decrease in stroke volume in presence of ODQ suggests the involvement of the soluble guanylate cyclase-cGMP axis. Due to the oxidant properties of ODQ [33], we cannot exclude that this inhibitor interferes with the reductase ability of tissue heme proteins, thus limiting the conversion of nitrite to NO. An important intramyocardial target of cGMP is PKG [34,35]. The finding that KT5823, an inhibitor structurally unrelated to cGMP, abolished the nitrite-induced inotropism in eel and frog is consistent with a cGMP-PKG-dependent mechanism. Interestingly, also in the rat heart nitrite effects were abolished by PGK inhibitor [36, this issue]. In both eel and frog hearts [34], PKG, as shown in the mammalian myocardium, may reduce L-type Ca^{2+} currents [37] and through troponin I phosphorylation reduces troponin C affinity for Ca^{2+}, thus negatively affecting contractility [38]. We suggest that a reduction of L-type Ca^{2+} current and a PKG-mediated myofilament desensitization to Ca^{2+} may contribute to the nitrite-induced negative inotropism in eel and frog.

Using the biotin switch method for the specific tagging of S-nitrosylated proteins [19], we evaluated the level of nitrosylated proteins in the hearts of eel and frog treated with nitrite. Protein S-nitrosylation, the covalent adduction of a nitroso group to a cysteine thiol side chain, has recently emerged as a major mechanism by which NO mediates a large number of intracellular processes [39]. Our results showed that nitrite-derived NO induces an increase of protein S-nitrosylation in both membrane and cytosolic fractions of frog and in membrane fraction of eel. A study in our laboratory is in progress to identify the final molecular targets of this nitrosylation process and their subcellular localization.

Recent findings have shown that S-nitrosylation regulates diverse pathways, including G-protein-coupled receptor signalling [40,41],

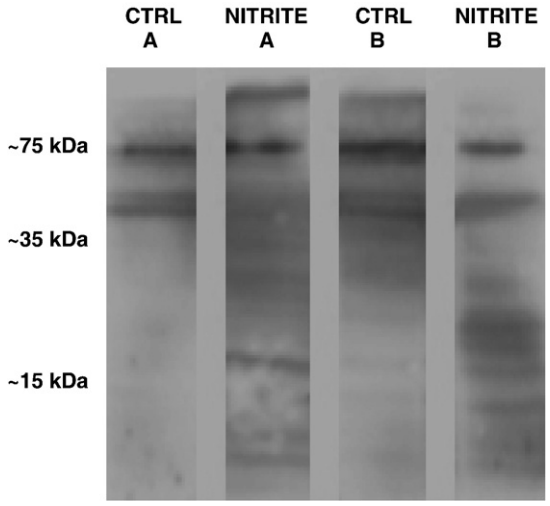

Fig. 6. Western blot analysis of S-nitrosylated proteins in frog heart homogenates. Cytosolic (A) and membrane protein fractions (B). CTRL: control; NITRITE: nitrite treated-hearts.

Table 2
Different levels of cardiac organization in the vertebrates species examined.

Species	Icefish, frog	Eel
Ventricular myoarchitecture	Trabecular	Trabecular + compact subepicardium
Type of blood supply	Venous, lacunary	Coronary to compact subepicardium, lacunary to innermost myocardium
Interface	EE	EE and VE

EE = endocardial-endothelium; VE = vascular endothelium; Ref. in [49].

Hsp90, a chaperone involved in eNOS activation [42] death receptor-mediated signalling [43], and mitochondrial pro- and anti- apoptotic cascades [44]. Moreover, dysregulated S-nitrosylation of the ryanodine receptor (Ca^{2+}-release channel) may contribute to cardiac arrhythmias [45], heat stroke [46], and impaired exercise capacity [47]. In this view, mitochondria deserve particular attention, also in light of the inhibition exerted by nitrite-derived NO on mitochondrial respiration observed by Shiva et al. [23] in both heart homogenate and isolated cardiomyocytes. The present study does not elucidate whether the negative inotropism observed in eel and frog relates to an inhibited mitochondrial respiration. Additional studies are needed in order to clarify whether in the heart of eel, icefish and frog, mitochondria are the final target of nitrite-derived NO. In this context, it should be underlined that, as shown by Shiva et al. [23], in the rat heart nitrite-derived NO reduces mitochondrial respiration through cytochrome *c* oxidase.

4.1. Conclusions and perspectives

We have shown in non-mammalian hearts with diverse morphologies and blood supply that, under normoxic conditions, nitrite modulates contractility and is also an important intracardiac source of NO. The different nitrite-dependent inotropic responses, which parallel those elicited by authentic NO, uncover at the heart level the already observed species-specific sensitivity to NO_2^-, well documented both in freshwater and marine teleosts [25], as well as in several amphibian species [48] after exposure to ambient nitrite. We argue that the comparative approach using different cardiac designs, such as those employed in this work, provides not only remarkable insights on "ancestral" functions of the nitrite-NO system in vertebrates, but also may help to expand its actual significance in human physiology. Indeed, these natural heart models represent powerful research systems that complement the more traditional models of human diseases, like the mouse, holding important clues for understanding fundamental cardiovascular mechanisms. For example, the phylogenetic relationship among Hb-expressing and Hb-less fish families constitutes an ideal matrix of genetic knockouts that can be employed to probe several challenging questions pertinent to the redox biome, including the newly discovered role of Hb and Mb as the predominant nitrite reductases able to generate and regulate NO signalling. By using the heart of the icefish *C. hamatus*, natural knock-out for Hb, and an Hb-free perfusion buffer, we demonstrated that Hb is not involved in the reduction of nitrite to NO in the myocardial tissue, thus corroborating the theory [23] that in the heart myoglobin may be the predominant nitrite reductase. In conclusion, comparative studies may pave the way towards an integrated systemic approach concerned with the role of nitrite/NO signalling in vertebrate cardiac physiology, which is also essential for medically-oriented studies and clinical applications.

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References

[1] P. Kleinbongard, A. Dejam, T. Lauer, T. Rassaf, A. Schindler, O. Picker, T. Scheeren, A. Gödecke, J. Schrader, R. Schulz, G. Heusch, G.A. Schaub, N.S. Bryan, M. Feelisch, M. Kelm, Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals, Free Radic. Biol. Med. 35 (2003) 790–796.

[2] K. Cosby, K.S. Partovi, J.H. Crawford, R.P. Patel, C.D. Reiter, S. Martyr, B.K. Yang, M.A. Waclawiw, G. Zalos, X. Xu, K.T. Huang, H. Shields, D.B. Kim-Shapiro, A.N. Schechter, R.O. Cannon, M.T. Gladwin, Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation, Nat. Med. 9 (2003) 1498–1505.

[3] M.T. Gladwin, J.H. Shelhamer, A.N. Schechter, M.E. Pease-Fye, M.A. Waclawiw, J.A. Panza, F.P. Ognibene, R.O. Cannon, Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 11482–11487.

[4] J. Rodriguez, R.E. Maloney, T. Rassaf, N.S. Bryan, M. Feelisch, Chemical nature of nitric oxide storage forms in rat vascular tissue, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 336–341.

[5] J.O. Lundberg, Nitric oxide metabolites and cardiovascular disease: markers, mediators, or both? J. Am. Coll. Cardiol. 47 (2006) 580–581.

[6] M.T. Gladwin, J.H. Crawford, R.P. Patel, The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation, Free Radic. Biol. Med. 36 (2004) 707–717.

[7] C. Gautier, E. van Faassen, I. Mikula, P. Martasek, A. Slama-Schwok, Endothelial nitric oxide synthase reduces nitrite anions to NO under anoxia, Biochem. Biophys. Res. Commun. 341 (2006) 816–821.

[8] M.T. Gladwin, D.B. Kim-Shapiro, The functional nitrite reductase activity of the heme-globins, Blood 112 (2008) 2636–2647.

[9] M.T. Gladwin, A.N. Schechter, D.B. Kim-Shapiro, R.P. Patel, N. Hogg, S. Shiva, R.O. Cannon, M. Kelm, D.A. Wink, M.G. Espey, E.H. Oldfield, R.M. Pluta, B.A. Freeman, J.R. Lancaster, M. Feelisch, J.O. Lundberg, The emerging biology of the nitrite anion, Nat. Chem. Biol. 1 (2005) 308–314.

[10] M.R. Duranski, J.J. Greer, A. Dejam, S. Jaganmohan, N. Hogg, W. Langston, R.P. Patel, S.F. Yet, X. Wang, C.G. Kevil, M.T. Gladwin, D.J. Lefer, Cytoprotective effects of nitrite during *in vivo* ischemia–reperfusion of the heart and liver, J. Clin. Invest. 115 (2005) 1232–1240.

[11] A. Webb, R. Bond, P. McLean, R. Uppal, N. Benjamin, A. Ahluwalia, Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia–reperfusion damage, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 13683–13688.

[12] N.S. Bryan, B.O. Fernandez, S.M. Bauer, M.F. Garcia-Saura, A.B. Milsom, T. Rassaf, R.E. Maloney, A. Bharti, J. Rodriguez, M. Feelisch, Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues, Nat. Chem. Biol. 1 (2005) 290–297.

[13] F.B. Jensen, Nitrite oxide formation from nitrite in zebrafish, J. Exp. Biol. 210 (2007) 3387–3394.

[14] B. Tota, R. Acierno, C. Agnisola, Mechanical performance of the isolated and perfused heart of the haemoglobinless Antarctic icefish *Chionodraco hamatus* (Lönnberg): effects of loading conditions and temperature, Philos. Trans. Soc. Lond. 332 (1991) 191–198.

[15] C.H. Cheng, H.W. Detrich 3rd, Molecular ecophysiology of Antarctic notothenioid fishes, Philos. Trans. R. Soc. Lond., B. Biol. Sci. 362 (2007) 2215–2232.

[16] S. Imbrogno, L. De Iuri, R. Mazza, B. Tota, Nitric oxide modulates cardiac performance in the heart of *Anguilla anguilla*, J. Exp. Biol. 204 (2001) 1719–1727.

[17] D. Pellegrino, C.A. Palmerini, B. Tota, No hemoglobin but NO: the icefish (*Chionodraco hamatus*) heart as a paradigm, J. Exp. Biol. 207 (2004) 3855–3864.

[18] A. Gattuso, R. Mazza, D. Pellegrino, B. Tota, Endocardial endothelium mediates luminal ACh-NO signaling in isolated frog heart, Am. J. Physiol. 276 (1999) H633–H641.

[19] S.R. Jaffrey, S.H. Snyder, The biotin switch method for the detection of S-nitrosylated proteins, Sci. STKE 86 (2001) PL1.

[20] G.F. Holeton, Oxygen uptake and circulation by a hemoglobinless Antarctic fish (*Chaenocephalus aceratus lonnberg*) compared with three red-blooded Antartic fish, Comp. Biochem. Physiol. 34 (1970) 457–471.

[21] B. Tota, D. Pellegrino, S. Shiva, T. Angelone, M.T. Gladwin, Physiological concentrations of nitrite modulate contractility in the vertebrate heart, Second International Meeting "Role of Nitrite in Physiology, Pathophysiology and Therapeutics" (NIH, Bethesda, USA) September 6–7, 2007.

[22] S. Aggergaard, F.B. Jensen, Cardiovascular changes and physiological response during nitrite exposure in rainbow trout, J. Fish Biol. 59 (2001) 13–27.

[23] S. Shiva, S. Huang, R. Grubina, L.A. Ringwood, P. MacArthur, X. Xu, V.M. Darley-Usmar, M.T. Gladwin, Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration, Circ. Res. 100 (2007) 654–661.

[24] D. Amelio, F. Garofano, D. Pellegrino, F. Giordano, B. Tota, M.C. Cerra, Cardiac expression and distribution of nitric oxide synthases in the ventricle of the cold-adapted Antarctic teleosts, the hemoglobinless *Chionodraco hamatus* and the red-blooded *Trematomus bernacchii*, Nitric Oxide 15–3 (2006) 190–198.

[25] F.B. Jensen, Nitrite disrupts multiple physiological functions in aquatic animals, Comp. Biochem. Physiol. 135A (2003) 9–24.

[26] R. Fischmeister, L. Castro, A. Abi-Gerges, F. Rochais, G. Vandecasteele, Species- and tissue-dependent effects of NO and cyclic GMP on cardiac ion channels, Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 142 (2005) 136–143.

[27] R. Grocott-Mason, S. Fort, M.J. Lewis, A.M. Shah, Myocardial relaxant effect of exogenous nitric oxide in isolated ejecting hearts, Am. J. Physiol. 266 (1994) H1699–H1705.

[28] J.L. Balligand, Regulation of cardiac beta-adrenergic response by nitric oxide, Cardiovasc. Res. 43 (1999) 607–620.

[29] N. Paolocci, T. Katori, H.C. Champion, M.E. St John, K.M. Miranda, J. Fukuto, D.A. Wink, D.A. Kass, Positive inotropic and lusitropic effects of HNO/NO− in failing hearts: independence from beta-adrenergic signalling, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 5537–5542.

[30] M.T. Gladwin, N.J.H. Raat, S. Shiva, C. Dezfulian, N. Hogg, D.B. Kim-Shapiro, R.P. Patel, Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation, Am. J. Physiol. 291 (2006) H2026–H2035.

[31] R.S. Wu, Hypoxia: from molecular responses to ecosystem responses, Mar. Pollut. Bull. 45 (2002) 35–45.

[32] R.G. Boutilier, P.H. Donohoe, G.J. Tattersall, T.G. West, Hypometabolic homeostasis in overwintering aquatic amphibians, J. Exp. Biol. 200 (1997) 387–400.

[33] M. Feelisch, P. Kotsonis, J. Siebe, B. Clement, H.H. Schmidt, The soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a] quinoxalin-1-one is a nonselective heme protein inhibitor of nitric oxide synthase and other cytochrome P-450 enzymes involved in nitric oxide donor bioactivation, Mol. Pharmacol. 56 (1999) 243–253.

[34] S. Imbrogno, T. Angelone, A. Corti, C. Adamo, K.B. Helle, B. Tota, Influence of vasostatins, the chromogranin A-derived peptides, on the working heart of the eel (*Anguilla anguilla*): negative inotropy and mechanism of action, Gen. Comp. Endocrinol. 139 (2004) 20–28.

[35] P.F. Méry, S.M. Lohmann, U. Walter, R. Fischmeister, Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 1197–1201.

[36] D. Pellegrino, S. Shiva, T. Angelone, M.T. Gladwin, B. Tota, Nitrite exerts potent negative inotropy in the isolated heart via eNOS-independent nitric oxide generation and cGMP–PKG pathway activation, Biochim. Biophys. Acta (2009) 818–827.

[37] N. Abi-Gerges, R. Fischmeister, P.F. Mery, G protein-mediated inhibitory effect of a nitric oxide donor on the L-type Ca^{2+} current in rat ventricular myocytes, J. Physiol. 531 (2001) 117–130.

[38] L. Hove-Madsen, P.F. Mery, J. Jurevicius, A.V. Skeberdis, R. Fischmeister, Regulation of myocardial calcium channels by cyclic AMP metabolism, Basic Res. Cardiol. 91 (1996) 1–8.

[39] D.T. Hess, A. Matsumoto, S.O. Kim, H.E. Marshall, J.S. Stamler, Protein S-nitrosylation: purview and parameters, Nat. Rev., Mol. Cell Biol. 6 (2005) 150–166.

[40] E. Nozik-Grayck, E.J. Whalen, J.S. Stamler, T.J. McMahon, P. Chitano, C.A. Piantadosi, S-nitrosoglutathione inhibits alpha1-adrenergic receptor-mediated vasoconstriction and ligand binding in pulmonary artery, Am. J. Physiol., Lung Cell. Mol. Physiol. 290 (2006) L136–L143.

[41] E.J. Whalen, M.W. Foster, A. Matsumoto, K. Ozawa, J.D. Violin, L.G. Que, C.D. Nelson, M. Benhar, J.R. Keys, H.A. Rockman, W.J. Koch, Y. Daaka, R.J. Lefkowitz, J.S. Stamler, Regulation of beta-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2, Cell 129 (2007) 511–522.

[42] A. Martinez-Ruiz, L. Villanueva, C. Gonzalez de Orduna, D. Lopez-Ferrer, S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 8525–8530.

[43] M. Benhar, M.T. Forrester, D.T. Hess, J.S. Stamler, Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins, Science 320 (2008) 1050–1054.

[44] B.M. Choi, H.O. Pae, S.I. Jang, Y.M. Kim, H.T. Chung, Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator, J. Biochem. Mol. Biol. 35 (2002) 116–126.

[45] D.R. Gonzalez, F. Beigi, A.V. Treuer, J.M. Hare, Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 20612–20617.

[46] W.J. Durham, P. Aracena-Parks, C. Long, A.E. Rossi, S.A. Goonasekera, S. Boncompagni, D.L. Galvan, C.P. Gilman, M.R. Baker, N. Shirokova, F. Protasi, R. Dirksen, S.L. Hamilton, RyR1 S-nitrosylation underlies environmental heat stroke and sudden death in Y522S RyR1 knockin mice, Cell 133 (2008) 53–65.

[47] S.E. Lehnart, M. Mongillo, A. Bellinger, N. Lindegger, B.X. Chen, W. Hsueh, S. Reiken, A. Wronska, L. Drew, C.W. Ward, W.J. Lederer, R.S. Kass, G. Morley, A.R. Marks, Leaky Ca^{2+} release channel/ryanodine receptor 2 causes seizures and sudden cardiac death in mice, J. Clin. Invest. 118 (2008) 2230–2245.

[48] C. Shinn, A. Marco, L. Serrano, Inter- and intra-specific variation on sensitivity of larval amphibians to nitrite, Chemosphere 71 (2008) 507–514.

[49] B. Tota, V. Cimini, G. Salvatore, G. Zummo, Comparative study of the arterial and lacunary systems of the ventricular myocardium of elasmobranch and teleost fishes, Am. J. Anat. 167 (1983) 15–32.